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AUTHOR(S):

Tanaka, Takashi; Kanatsu-Shinohara, Mito; Lei, Zhenmin; Rao, C.V.; Shinohara, Takashi

CITATION:

Tanaka, Takashi ...[et al]. The Luteinizing Hormone-Testosterone Pathway Regulates Mouse Spermatogonial Stem Cell Self-Renewal by Suppressing WNT5A Expression in Sertoli Cells. Stem Cell Reports 2016, 7(2): 279-291

ISSUE DATE:

2016-08-09

URL:

http://hdl.handle.net/2433/216286

Stem Cell Reports
Article
ISSCR
OPEN ACCESS

The Luteinizing Hormone-Testosterone Pathway Regulates Mouse Spermatogonial Stem Cell Self-Renewal by Suppressing WNT5A Expression in Sertoli Cells

Takashi Tanaka,[1] Mito Kanatsu-Shinohara,[1,2] Zhenmin Lei,[3] C.V. Rao,[4] and Takashi Shinohara[1,*]
[1]Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
[2]Japan Science and Technology Agency, PRESTO, Kyoto 606-8501, Japan
[3]Department of OB/GYN and Women's Health, University of Louisville School of Medicine, Louisville, KY 40292, USA
[4]Departments of Cellular Biology and Pharmacology, Molecular and Human Genetics, and Obstetrics and Gynecology, Reproduction and Development Program, Herbert Wertheim College of Medicine, Florida International University, Miami, FL 33199, USA
*Correspondence: tshinoha@virus.kyoto-u.ac.jp
http://dx.doi.org/10.1016/j.stemcr.2016.07.005

SUMMARY

Spermatogenesis originates from self-renewal of spermatogonial stem cells (SSCs). Previous studies have reported conflicting roles of gonadotropic pituitary hormones in SSC self-renewal. Here, we explored the role of hormonal regulation of SSCs using *Fshb* and *Lhcgr* knockout (KO) mice. Although follicle-stimulating hormone (FSH) is thought to promote self-renewal by glial cell line-derived neurotrophic factor (GDNF), no abnormalities were found in SSCs and their microenvironment. In contrast, SSCs were enriched in *Lhcgr*-deficient mice. Moreover, wild-type SSCs transplanted into *Lhcgr*-deficient mice showed enhanced self-renewal. Microarray analysis revealed that *Lhcgr*-deficient testes have enhanced WNT5A expression in Sertoli cells, which showed an immature phenotype. Since WNT5A was upregulated by anti-androgen treatment, testosterone produced by luteinizing hormone (LH) is required for Sertoli cell maturation. WNT5A promoted SSC activity both in vitro and in vivo. Therefore, FSH is not responsible for GDNF regulation, while LH negatively regulates SSC self-renewal by suppressing WNT5A via testosterone.

INTRODUCTION

Spermatogonial stem cells (SSCs) are the founder cell population of spermatogenesis (de Rooij and Russell, 2000; Meistrich and van Beek, 1993). Although their number in testes is very small (0.02%–0.03% of total germ cells), they are the only stem cells in the germline that have the unique ability to undergo self-renewal division to produce numerous progenitor cells. SSCs are thought to develop from gonocytes in the neonatal testis during the perinatal stage (Shinohara et al., 2001). SSCs rapidly expand their number during sexual maturation and maintain a constant population size in adults. The SSC population size is determined based on the number of niches (Oatley et al., 2011), which likely develop through complex interactions among Sertoli cells, Leydig cells, peritubular myoid cells, and macrophages (DeFalco et al., 2015; Meng et al., 2000; Oatley et al., 2009; Spinnler et al., 2010). Sexual maturation is accompanied by dynamic changes in hormonal milieu, and development of these cell types is influenced by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted from the pituitary gland. However, little is known regarding the roles of these hormones in SSC and niche development.

SSC research was revolutionized by the development of spermatogonial transplantation in 1994 (Brinster and Zimmermann, 1994). This technique provided the first functional assay for SSCs and has been widely used to characterize SSCs. In initial attempts to improve the colonization efficiency of SSCs, suppression of hypothalamus signaling was found to confer beneficial effects on transplantation. Treatment of recipient animals with leuprolide, a gonadotropin-releasing hormone (GnRH) analog, resulted in enhanced colonization of donor cells. The donor cell colony count increased by approximately 2.7-fold, which was suggestive of enhanced homing of SSCs (Ogawa et al., 1998). Moreover, leuprolide appeared to enhance self-renewal division because the colonized area in leuprolide-treated recipients increased by 5.3-fold (Dobrinski et al., 2001). Prolonged administration of leuprolide suppresses the secretion of LH and FSH from the pituitary gland, which induces testosterone production by Leydig cells and stimulates the proliferation of Sertoli cells, respectively (Karashima et al., 1988). Although a GnRH analog is generally suppressive to spermatogenesis in wild-type (WT) mice, it protects SSCs from damage by chemical or radiation treatment (Meistrich, 1998). This suppressive effect of the gonadotropic pituitary hormones on SSC self-renewal was confirmed in another study, which showed that SSCs underwent more extensive self-renewal divisions in hypophysectomized hosts (Kanatsu-Shinohara et al., 2004). Several possibilities, including the direct action of GnRH on germ cells, reduction of intratesticular testosterone, or increased kit ligand (KITL) expression, are believed to be involved. However, further studies are required (Ogawa et al., 1998). These studies performed

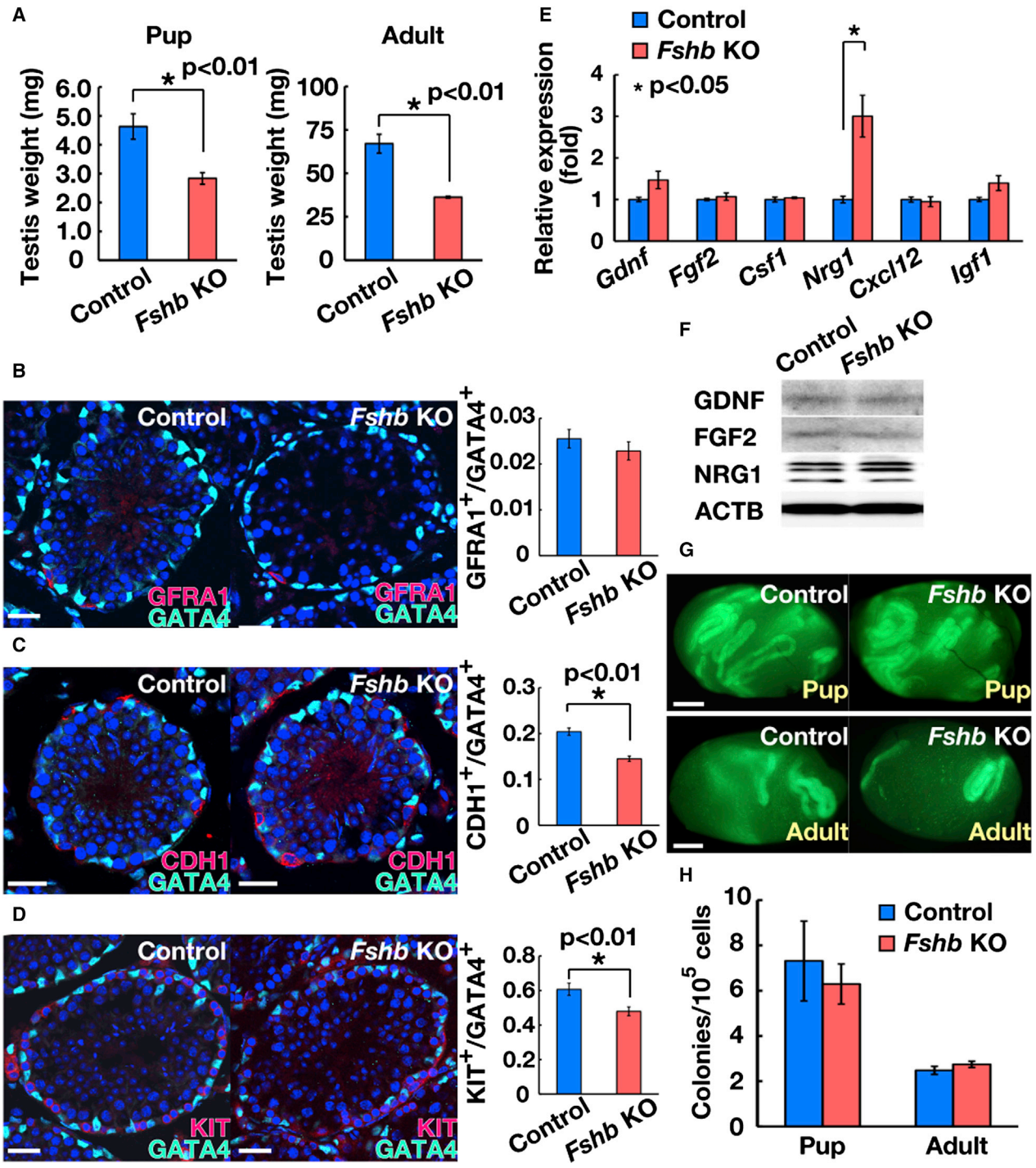

Figure 1. Functional Analysis of SSCs in *Fshb* KO Mice

(A) Testis weight of 8-day-old and 6-week-old mice (n = 4 testes).

(B–D) Immunohistochemistry and quantification of indicated spermatogonia markers in *Fshb* KO adult mouse testes. At least 200 cells in four testes were counted.

(E) Real-time PCR analysis of busulfan-treated adult mouse testes (n = 7 experiments).

(F) Western blot analysis of busulfan-treated adult mouse testes.

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more than a decade ago established the beneficial effects of suppressing gonadotropic pituitary hormones in promoting SSC self-renewal.

In 2000, glial cell line-derived neurotrophic factor (GDNF) was identified as an SSC self-renewal factor (Meng et al., 2000). GDNF is a member of the transforming growth factor β superfamily, which is expressed in Sertoli cells. Transgenic mice overexpressing *Gdnf* showed clumps of undifferentiated spermatogonia in seminiferous tubules, while *Gdnf* heterozygous knockout (KO) mice underwent depletion of spermatogonia, resulting in male infertility. These results suggest that the amount of GDNF influences the fate of SSCs. The fact that GnRH analog treatment increased the length of the colony suggests that GDNF is induced by suppression of the gonadotropic pituitary hormones. However, subsequent studies showed that GDNF expression in Sertoli cells is positively regulated by FSH (Tadokoro et al., 2002). In that study, the proliferation of undifferentiated spermatogonia was significantly reduced when FSH was depleted using a gonadotropin-releasing hormone antagonist (Nal-Glu). These testes showed reduced GDNF expression. Moreover, FSH (but not testosterone) increased GDNF expression in testis cell culture. Regulation of GDNF expression by FSH was also supported by another in vivo study that showed increases in *Gdnf* mRNA levels in testes of immature mice that had been treated with FSH (Ding et al., 2011). However, this FSH-mediated regulation of GDNF was not confirmed in a testis cell-culture system that can maintain SSCs for the long term without FSH (Kanatsu-Shinohara et al., 2012).

In addition to FSH-mediated regulation, more recent studies suggest the involvement of testosterone in GDNF expression. Although GDNF was thought to be expressed in Sertoli cells, it has been shown that GDNF is expressed in peritubular myoid cells in both mouse and human testes (Chen et al., 2014; Spinnler et al., 2010). Testosterone induced GDNF expression at the mRNA and protein levels in peritubular cells in vitro (Chen et al., 2014). THY1-expressing mouse spermatogonia, which are thought to be enriched for SSCs, produced more colonies by testosterone treatment when they were cultured with peritubular myoid cells. Males that lacked *Gdnf* in peritubular cells were initially fertile but lost undifferentiated spermatogonia over the long term (Chen et al., 2016). Thus, conflicting reports exist on the role of the gonadotropic pituitary hormones in SSC regulation, and our current understanding is apparently incomplete.

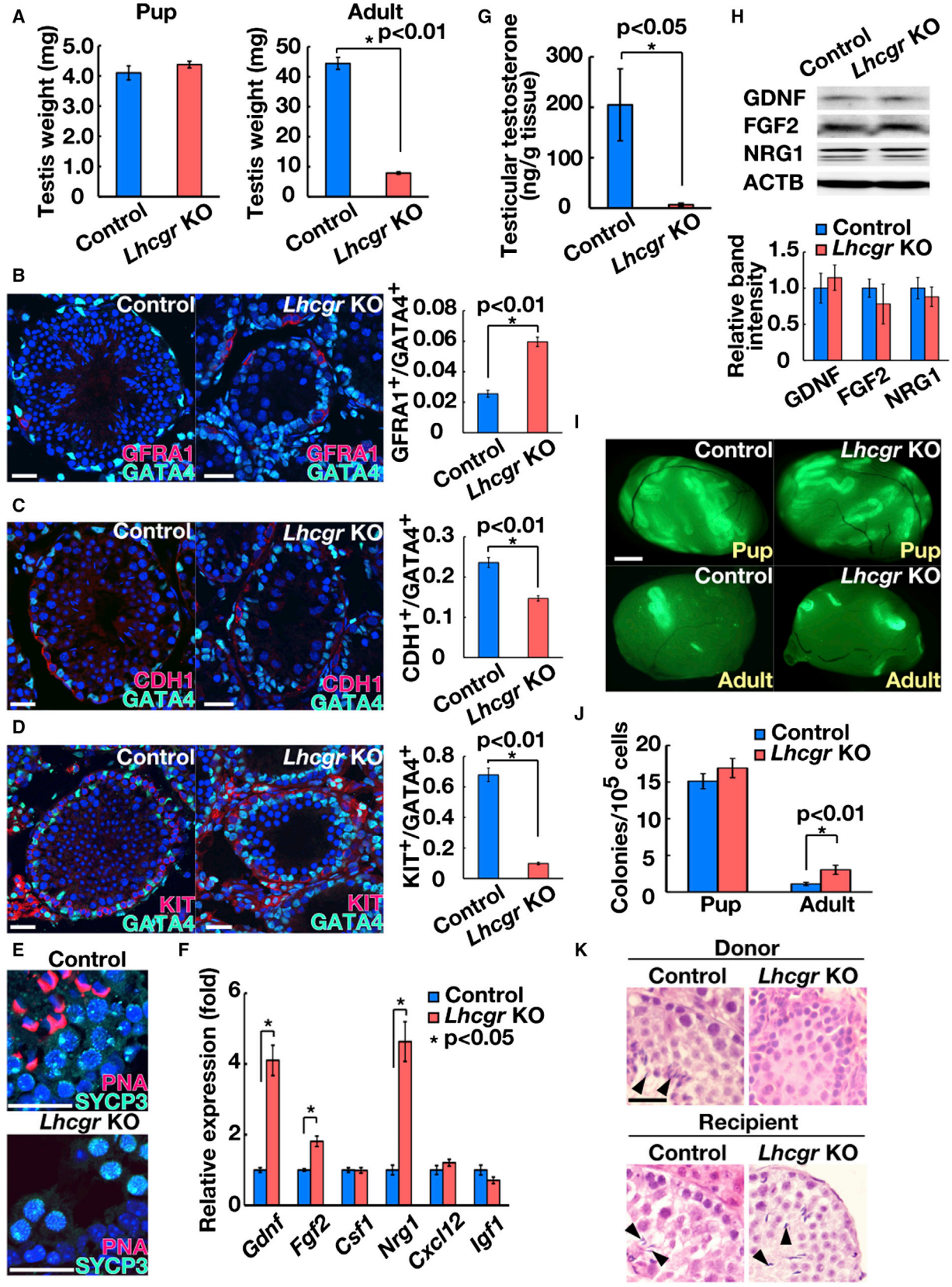
In this study, we examined the impact of hormonal signaling on SSC self-renewal using follicle-stimulating hormone β (*Fshb*; FSH β subunit) and luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) KO mice. FSH acts on Sertoli cells within seminiferous tubules, while LHCGR are detected only on the testosterone-producing Leydig cells located between the tubules. *Fshb* KO mice are fertile but have smaller testes with reduced Sertoli and germ cell numbers (Kumar et al., 1997). *Lhcgr* KO mice have undescended testes and are infertile (Lei et al., 2001; Zhang et al., 2001). SSC activities of immature and mature testes of these mutant mice were determined based on spermatogonial transplantation into WT mice. We also examined the effect of mutant testicular microenvironments on SSC homing and self-renewal division by serial transplantation. Microarray analysis revealed that *Wnt5a* is involved in SSC self-renewal by hormonal signaling.

RESULTS

Phenotypic and Functional Analysis of Spermatogonia in Fshb KO Mice

Because FSH has been implicated in the regulation of GDNF expression, we first used *Fshb* KO mice to examine the effect of this gene on SSCs (Kumar et al., 1997). Testis weight was significantly lower in both pup and adult *Fshb* KO mice than in the control at each stage (Figure 1A) ($p = 0.0073$ for pup; $p = 0.0059$ for adult), suggestive of abnormalities in differentiation. Immunohistochemical analysis of adult testis showed no significant changes in the number of cells expressing glial cell line-derived neurotrophic factor family receptor α1 (GFRA1; a marker for A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia) (Figure 1B). However, the number of cells expressing cadherin 1 (CDH1; a marker for undifferentiated spermatogonia) or Kit oncogene (KIT; a marker for differentiating spermatogonia) was significantly decreased (Figures 1C and 1D) ($p < 0.0001$ for CDH1; $p = 0.0037$ for KIT), suggesting that FSH may play a role in spermatogonia differentiation. We also examined the expression of several molecules involved in spermatogonia proliferation/fate in busulfan-treated testes based on real-time PCR. Although neuregulin 1 (*Nrg1*) was expressed more strongly in *Fshb* KO mice (Figure 1E) ($p = 0.0017$), western blot analysis showed no changes in NRG1 expression (Figure 1F). Neither GDNF nor fibroblast growth factor 2 (FGF2) showed significant changes by western blotting.

(G) Macroscopic appearance of recipient testes.
(H) Colony counts (n = 18 testes for pup, n = 16 testes for adult; three experiments).
Counterstain: Hoechst 33342 (B–D). Scale bars represent 25 μm (B–D) and 1 mm (G). Error bars indicate SEM. Asterisks indicate statistical significance. See also Tables S2 and S3.



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Although these results indicate that undifferentiated spermatogonia are not influenced by the absence of FSH signaling, SSCs are defined by their function and comprise a small number among undifferentiated spermatogonia. Therefore, the effects on SSCs could not be determined based on morphology alone. To clarify this point, we performed spermatogonial transplantation using pup and adult testes and examined their SSC activity. *Fshb* KO mice were crossed with green mice to introduce a donor cell marker. Testis cells from pup and adult mice were transplanted into congenitally infertile WBB6F1-W/W^{v} mice (W mice) to determine the SSC activity.

Analyses of recipient mice at 2 months post transplantation revealed that comparable numbers of germ cell colonies were generated from *Fshb* KO and WT testes regardless of age (Figure 1G). The numbers of colonies from *Fshb* KO and WT pup testis cells were 6.3 and 7.3 per 10^5 cells, respectively (n = 18). Likewise, the numbers of colonies from *Fshb* KO and WT adult testis cells were 2.7 and 2.3 per 10^5 cells, respectively (n = 16) (Figure 1H). Histological analysis of recipient testes also showed similar levels of colonization from both *Fshb* KO and control mice regardless of the donor age (data not shown). These results suggest that the absence of FSH signaling does not influence the development of SSCs in postnatal testis, although immunohistochemical analysis is suggestive of abnormalities in differentiation of committed spermatogonia.

Phenotypic and Functional Analysis of Spermatogonia in Lhcgr KO Mice

In the second set of experiments, we used *Lhcgr* KO mice and examined their SSC activity (Lei et al., 2001). Although the testis weight of *Lhcgr* KO pups was comparable with that of control mice, it was significantly reduced in adults (Figure 2A) ($p = 0.00013$), suggesting that *Lhcgr* deficiency caused testis abnormalities distinct from those observed in *Fshb* mice. Consistent with this hypothesis, immunohistochemical analysis of adult testes revealed that *Lhcgr* KO mice have more GFRA1-expressing spermatogonia than controls (Figure 2B) ($p < 0.0001$). However, the number of CDH1^{+} and KIT^{+} spermatogonia was reduced in these mice (Figures 2C and 2D) ($p < 0.0001$ for CDH1; $p < 0.0001$ for KIT), suggesting that the total undifferentiated spermatogonia population and differentiating spermatogonia population are smaller than those in WT mice. Moreover, although meiotic cells were found, no elongated spermatids developed in these mice (Figure 2E), which was consistent with our previous study (Lei et al., 2004). Because GFRA1 is thought to be expressed in SSCs (Kanatsu-Shinohara and Shinohara, 2013), these results suggest that *Lhcgr* deficiency is accompanied by increases in SSCs and decreases in differentiating spermatogonia. To explore the mechanism underlying this observation, we performed real-time PCR and examined busulfan-treated mouse testes that do not have germ cells. Although testosterone is thought to increase GDNF expression in testis (Chen et al., 2016), our analyses revealed significantly increased expression of several candidate mRNAs, including those of *Gdnf Fgf2* and *Nrg1*, in *Lhcgr* KO mice (Figure 2F) ($p < 0.0001$ for *Gdnf*; $p < 0.0001$ for *Fgf2*; $p < 0.0001$ for *Nrg1*). This occurred despite the significantly reduced production of testosterone (Figure 2G) ($p = 0.0023$). However, we did not observe an increase in the protein level based on western blot analysis (Figure 2H).

We used pup and adult testes and quantified the numbers of SSCs based on spermatogonial transplantation. These transplantation experiments showed a comparable number of colonies between testis cells prepared from immature *Lhcgr* KO and WT mice (Figure 2I). The number of colonies generated by 10^5 *Lhcgr* KO and control pup testis cells were 16.9 and 15.1, respectively (n = 14) (Figure 2J). However, as expected from the immunohistochemical staining, significantly more colonies were found in adult *Lhcgr* KO mice. The number of colonies generated by 10^5 *Lhcgr* KO and control adult testis cells was 3.0 and 1.1, respectively (n = 18), showing approximately 2.7-fold SSC enrichment in *Lhcgr* KO mice ($p = 0.0071$). Transplantation experiments also rescued the abnormal spermatogenesis of *Lhcgr*

Figure 2. Functional Analysis of SSCs in *Lhcgr* KO Mice

(A) Testis weight of 8-day-old and 6-week-old mice (n = 4 testes).

(B–D) Immunohistochemistry and quantification of indicated spermatogonia markers in *Lhcgr* KO adult mouse testes. At least 200 cells in four testes were counted.

(E) Immunohistochemistry of meiotic (SYCP3) and haploid (peanut agglutinin, PNA) cell markers.

(F) Real-time PCR analysis of busulfan-treated adult mouse testes (n = 8 testes). PCR was performed in triplicate.

(G) Intratesticular testosterone levels in untreated adult *Lhcgr* KO mice (n = 7 testes).

(H) Western blot analysis and quantification of GDNF, FGF2, and NRG1 in busulfan-treated adult mouse testes (n = 6 testes).

(I) Macroscopic appearance of recipient testes.

(J) Colony counts (n = 14 testes for pup, n = 18 testes for adult; three experiments).

(K) Histology of donor and recipient testes. Arrowheads indicate elongated spermatids.

Counterstain: Hoechst 33342 (B–E). Scale bars represent 25 μm (B–E and K), and 1 mm (I). Error bars indicate SEM. Asterisks indicate statistical significance. See also Tables S2 and S3.

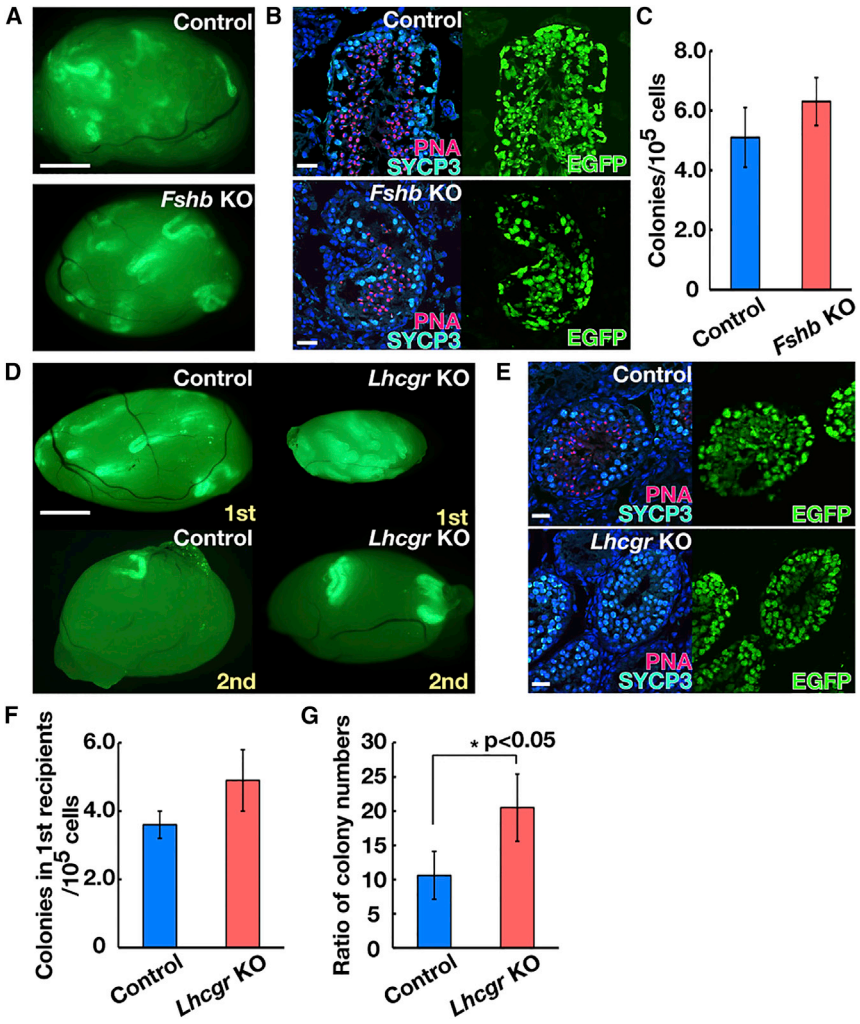

Figure 3. Transplantation of Green Mouse Testis Cells into Busulfan-Treated KO Mouse Recipient Testes

(A) Macroscopic appearance of *Fshb* KO recipient testes.

(B) Immunohistochemistry of *Fshb* KO recipient testes using meiotic (SYCP3) and haploid (PNA) cell markers.

(C) Colony counts (n = 18 testes for *Fshb* KO, n = 20 testes for control; three experiments).

(D) Macroscopic appearance of recipient testes using *Lhcgr* KO mice as primary recipients for serial transplantation.

(E) Immunohistochemistry of *Lhcgr* KO recipient testes using meiotic (SYCP3) and haploid (PNA) cell markers.

(F) Colony counts (n = 18 testes for *Lhcgr* KO, n = 19 testes for control; three experiments).

(G) Ratio of colony numbers ([total regenerated colony number × 10]/[colony number in primary recipients]) (n = 15 testes for *Lhcgr* KO, n = 14 testes for control; three experiments). The difference was significant by one-tailed t test.

Counterstain: Hoechst 33342 (B and E). Error bars indicate SEM. Scale bars represent 1 mm (A and D) and 25 μm (B and E). Asterisk indicates statistical significance. See also Table S3.

KO germ cells. Although germ cell differentiation was arrested at the round spermatid stage in *Lhcgr* KO donor testis, elongated spermatids were found after transplantation in recipient testes (Figure 2K). These results indicate that SSCs are enriched in *Lhcgr* KO testes and suggest that arrested spermatogenic differentiation is caused by abnormalities in the testicular microenvironment.

Evaluation of the Testicular Microenvironment by Serial Transplantation

Although the results in the preceding section showed 2.7-fold enrichment of SSCs in *Lhcgr* KO testes, it remains unclear whether the enrichment was due to the reduced number of post-meiotic germ cells in these mice or increased self-renewal factor secretion. To explore these possibilities, we performed spermatogonial transplantation into the mutant microenvironment to examine the impact of the microenvironment (Figure 3A). We also used *Fshb* KO mice to explore whether lack of FSH influences SSC colonization. In these experiments, *Fshb* or *Lhcgr* KO mice were treated with busulfan to remove endogenous spermatogenesis. Testis cells from adult green mice were dissociated and transplanted into the seminiferous tubules of mutant and control testes to examine the impact of the testicular microenvironment on donor SSC self-renewal.

When the recipients were analyzed 2 months after transplantation, we did not observe significant changes in the number of colonies in both *Fshb* and *Lhcgr* KO mouse recipient testes. The numbers of colonies generated by 10^5 donor cells were 6.3 ± 1.0 and 5.1 ± 0.8 in *Fshb* KO and control recipient mice, respectively (n = 18 for *Fshb* KO; n = 20 for control) (Figures 3B and 3C). Likewise, the numbers of colonies generated by 10^5 donor cells were 4.9 ± 0.9 and 3.6 ± 0.4 in *Lhcgr* KO and control recipient mice, respectively (n = 18 for *Lhcgr* KO; n = 19 for control) (Figures 3D–3F); however, the differences in these numbers were not statistically significant. The lack of effects in *Fshb* and *Lhcgr* KO mice contrasted with previous studies using

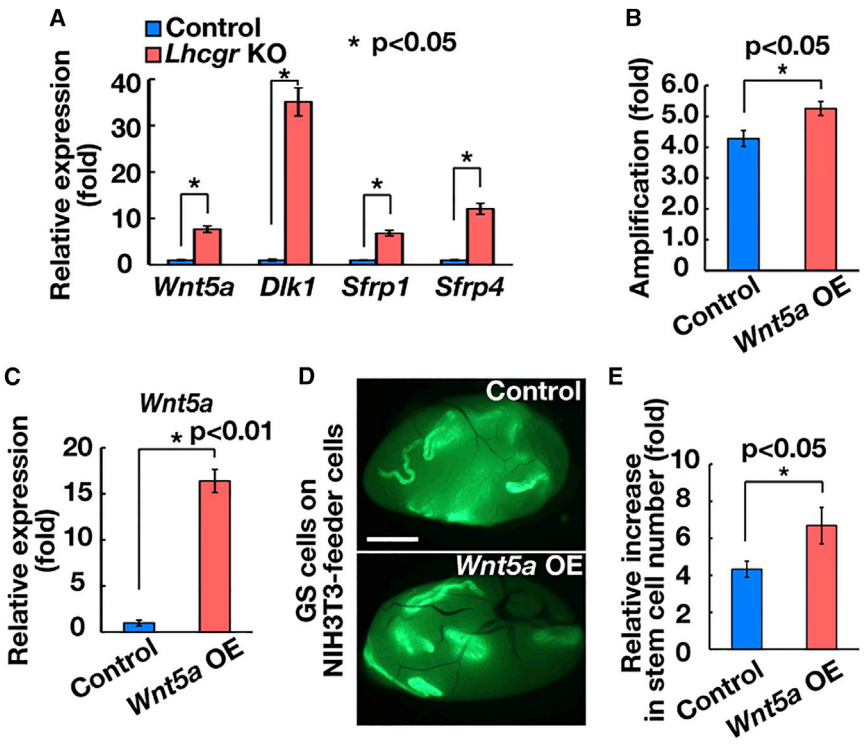

Figure 4. Effect of *Wnt5a* OE on SSC Self-Renewal In Vitro

(A) Real-time PCR analyses of busulfan-treated *Lhcgr* KO mouse testes (n = 8 experiments).

(B) Increased proliferation of GSCs cultured on NIH3T3 cells expressing *Wnt5a* (n = 6 experiments). Cells were recovered 7 days after culture initiation.

(C) Real-time PCR analysis of *Wnt5a* expression in NIH3T3 cells after *Wnt5a* OE (n = 4 experiments). Cells were recovered 4 days after transfection.

(D) Macroscopic appearance of recipient testes that received transplantation of GSCs cultured for 7 days on NIH3T3 cells after *Wnt5a* OE.

(E) Relative increase in SSC number after culture (n = 16 testes for *Wnt5a* OE, n = 17 testes for control; three experiments).

Error bars indicate SEM. Scale bar represents 1 mm (D). See also Tables S1 and S2.

GnRH analogs or hypophysectomized mice, which showed increased colony formation.

Although the results indicated no apparent changes in colony numbers in both mutants, the increased number of GFRA1$^+$ spermatogonia and enrichment of SSCs in *Lhcgr* KO mice were suggestive of enhanced SSC self-renewal in *Lhcgr* KO mouse testes. Therefore, we performed serial transplantation to explore whether the degree of self-renewal is improved in *Lhcgr* KO mouse recipient testes by dissociating the recipient testes and transplanting into secondary recipients using W mice. Assuming that each colony is derived from single SSCs, and that 10% of the injected cells colonize in the secondary recipients (Kanatsu-Shinohara et al., 2006; Nagano et al., 1999), the ratio of SSC number between the two time points was 20.5 ± 4.9 and 10.6 ± 3.5 in *Lhcgr* KO and control recipients, respectively (n = 15 for *Lhcgr* KO; n = 14 for control) (Figure 3G). The difference between the *Lhcgr* KO and control recipients was statistically significant ($p = 0.028$, one-tailed t test). These results suggested that SSCs that had been transplanted in *Lhcgr* KO mice underwent more extensive self-renewal divisions.

Increased Expression of Wingless-type MMTV Integration Site Family Member 5A in Lhcgr KO Mice

Because GDNF protein levels did not change in *Lhcgr* KO mice, we considered it likely that other genes are involved in enhanced SSC self-renewal. To explore this possibility, we used busulfan-treated *Lhcgr* KO and control WT mice and examined their gene-expression patterns using microarray analysis. Comparison between *Lhcgr* KO and control mice showed increased expression of 101 genes in *Lhcgr* KO mice, while 385 genes were downregulated (>2-fold change) (Table S1). Of these, we searched for cytokines or secreted molecules implicated in cell proliferation and identified several candidate molecules, including wingless-type MMTV integration site family member 5A (*Wnt5a*), delta-like 1 homolog (*Dlk1*), secreted frizzled-related protein 1 (*Sfrp1*), and secreted frizzled-related protein 4 (*Sfrp4*), which were upregulated in *Lhcgr* KO mice. The increased expression of these candidate genes was also confirmed based on real-time PCR, which showed increased expression of *Wnt5a*, *Dlk1*, *Sfrp1*, and *Sfrp4* by 7.7-, 35.1-, 6.8-, and 12.1-fold, respectively (Figure 4A) ($p < 0.0001$).

To examine the effect of these genes on SSC self-renewal, we used germline stem cells (GSCs), cultured spermatogonia with enriched SSC activity (Kanatsu-Shinohara et al., 2003). Candidate genes were transfected into NIH3T3 cells using a lentivirus vector, and GSCs were cultured for 7 days on these virus-infected feeder cells. Although proliferation of GSCs on NIH3T3 cells transfected with *Dlk1*, *Sfrp1*, or *Sfrp4* did not show significant differences from controls, those on *Wnt5a*-transfected cells showed increased recovery (Figures 4B and 4C) ($p = 0.018$ for amplification; $p < 0.0001$ for

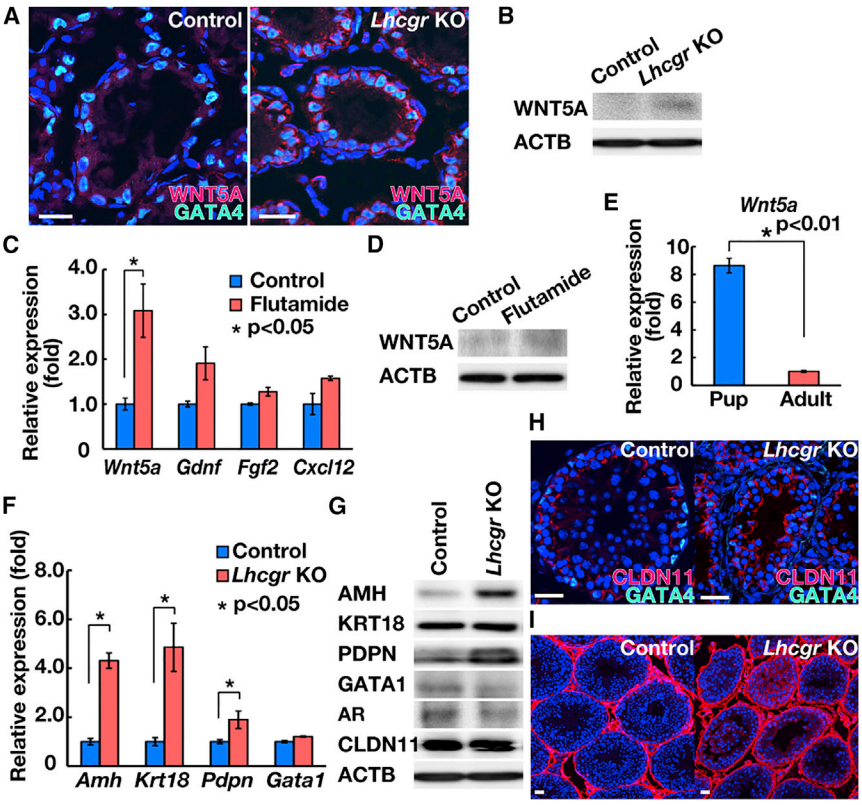

Figure 5. Immaturity of Sertoli Cells in *Lhcgr* KO Mice

(A) Immunohistochemistry of WNT5A expression in busulfan-treated *Lhcgr* KO mice.

(B) Western blot analysis of busulfan-treated *Lhcgr* KO mouse testes.

(C and D) Real-time PCR analysis (C; n = 4 experiments) and western blot (D) of busulfan-treated WT testes after flutamide administration. Testes were recovered 2 weeks after the flutamide treatment.

(E) Real-time PCR analysis of *Wnt5a* expression in 7-day-old and 6-week-old WT testes (n = 4 experiments).

(F and G) Real-time PCR (F; n = 8 experiments) and western blot analyses (G) of Sertoli cell markers in busulfan-treated *Lhcgr* KO mouse testes.

(H) Immunohistochemistry of CLDN11 expression in *Lhcgr* KO testis.

(I) Functional assessment of BTB in *Lhcgr* KO mouse testes. *Lhcgr* KO mice were injected interstitially with biotin (red).

Counterstain: Hoechst 33,342. Scale bars represent 25 μm (A, H, and I). Error bars indicate SEM. Asterisks indicate statistical significance. See also Tables S2 and S3.

expression). Cell recovery after trypsin digestion was 1.2-fold greater on *Wnt5a*-secreting cells. Although the increase was very minor, the difference was statistically significant. These results suggested that *Wnt5a* promotes GSC proliferation.

Since *Wnt5a* appears to play a role in the promotion of SSC self-renewal in *Lhcgr* KO mice, we examined the expression of WNT5A in vivo by immunohistochemistry. WNT5A was barely expressed in busulfan-treated control testis, while it was strongly detected in *Lhcgr* KO mice (Figure 5A). As expected from real-time PCR, *Lhcgr* KO mice showed stronger WNT5A expression based on western blotting (Figure 5B). Because androgens are produced by LH stimulation from Leydig cells, we examined whether androgens are involved in *Wnt5a* expression. Busulfan-treated mice were exposed to flutamide, a synthetic non-steroidal anti-androgen that acts by binding to an androgen receptor (Tohda et al., 2001). When their testes were recovered 2 weeks after treatment, increased expression was demonstrated by both real-time PCR and western blotting (Figures 5C and 5D) ($p = 0.031$). These results suggest that androgens mediate the suppression of WNT5A expression from Sertoli cells.

Although *Wnt5a* expression was very weak in WT adult testes, we noted that this gene was expressed more strongly in pup testes (Figure 5E) ($p < 0.0001$). Because this result suggests that Sertoli cells in *Lhcgr* KO mice exhibit an immature phenotype, we examined the expression of other immature Sertoli cell markers. As expected, anti-Müllerian hormone (AMH) and podoplanin (PDPN) were upregulated in *Lhcgr* KO testes (Figures 5F and 5G) ($p < 0.0001$ for *Amh*; $p = 0.0017$ for *Krt18*; $p = 0.028$ for *Pdpn*). On the other hand, adult Sertoli cell markers, such as GATA binding protein 1 (GATA1) or androgen receptor (AR), were only weakly expressed in *Lhcgr* KO testes. However, this does not necessarily support that Sertoli cells are completely immature because keratin 18 (KRT18), another marker of immature Sertoli cells, did not show apparent changes, and we could not identify cells stained with proliferating cell nuclear antigen or antigen identified by monoclonal antibody Ki67 (MKI67). These findings suggest that Sertoli cells in *Lhcgr* KO mice are mitotically quiescent.

Because immunohistochemical staining was suggestive of an immature phenotype, we further examined the effect of *Lhcgr* deficiency on the blood-testis barrier (BTB), which is found only in mature testes. Although claudin 11 (CLDN11) expression was clearly observed between Sertoli cells (Figure 5H), injection of biotin into the interstitium of untreated mature *Lhcgr* KO mouse testes resulted in leakage into the adluminal compartment, whereas such leakage was not observed in control WT mice (Figure 5I). These phenotypic and functional analyses suggest that Sertoli

cells in *Lhcgr* KO mice are phenotypically immature and have a defective BTB.

Enhanced Self-Renewal Division by Wnt5a

To confirm whether WNT5A promotes SSC self-renewal, we performed spermatogonial transplantation. This was because only 1%–2% of GSCs showed SSC activity, and it was possible that increased proliferation was due to enhanced progenitor cell proliferation (Kanatsu-Shinohara and Shinohara, 2013). We transplanted cells before and after culturing on NIH3T3 cells. Analysis of recipients showed that the number of SSCs was greater when GSCs on *Wnt5a*-expressing NIH3T3 cells were transplanted (Figures 4D and 4E). The number of colonies generated by GSCs on *Wnt5a*-expressing cells and control cells was 318.8 and 250.6 per 10^5 cells, respectively (n = 16 for *Wnt5a*; n = 17 for control). There was no significant difference in the number of colonies. However, while SSCs on control NIH3T3 cells increased by 4.3 ± 0.4-fold (n = 16), those on *Wnt5a*-expressing cells increased by 6.7 ± 1.0-fold (n = 17), showing a 1.5-fold increase by *Wnt5a* stimulation (p = 0.032), suggesting that the effect of *Wnt5a* is promoting proliferation, and not the rescue of apoptosis. These results confirm the beneficial effect of WNT5A on SSC self-renewal in vitro.

Because in vitro culture experiments showed increased self-renewal division of SSCs by *Wnt5a*, we examined the impact of *Wnt5a* in vivo. We transfected *Wnt5a* in Sertoli cells of busulfan-treated mice and examined the effect of this transfection on colony number after spermatogonial transplantation (Figure 6A). We microinjected a lentivirus-expressing *Wnt5a* into the seminiferous tubules, and donor testis cells from green mice were transplanted into seminiferous tubules 7 days after *Wnt5a* overexpression (OE; Figure 6B) (p = 0.046). Empty vector was transfected as a control. The numbers of colonies generated in *Wnt5a*-transfected or control mice were 6.1 ± 0.9 and 5.2 ± 0.8 per 10^5 cells, respectively (n = 8 for *Wnt5a*-treated; n = 10 for control) (Figures 6C and 6D). Although more colonies were observed in *Wnt5a*-transfected hosts, the difference was not statistically significant. These results suggest that *Wnt5a* expression does not have a significant effect on SSC migration into germline niches.

In the next set of experiments we microinjected *Wnt5a*-expressing lentivirus into testes of untreated adult green mice (Figure 6E). Testis cells were collected 2 months after *Wnt5a* OE (Figure 6F) (p = 0.0013), and dissociated testis cells were transplanted into the seminiferous tubules of busulfan-treated mice. The numbers of colonies generated by *Wnt5a*-transfected and control donor cells were 2.2 ± 0.4 and 1.0 ± 0.3 per 10^5 cells, respectively (n = 18 for *Wnt5a*-treated; n = 19 for control) (Figures 6G and 6H) (p = 0.009), suggesting that *Wnt5a* has a beneficial effect on SSC self-renewal in vivo. Taken together, these results suggest that LH suppresses SSC self-renewal by downregulating *Wnt5a* via androgens (Figure 6I).

DISCUSSION

Regulation of SSC self-renewal by gonadotropic hormones has been suggested since the early days of spermatogonial transplantation experiments. Several studies based on GnRH analog administration have demonstrated the involvement of the gonadotropic pituitary hormone in SSC regulation (Dobrinski et al., 2001; Ogawa et al., 1998), which was confirmed by another study using hypophysectomized animals (Kanatsu-Shinohara et al., 2004). However, the mechanism underlying this phenomenon has remained unclear. Moreover, controversy exists regarding the regulation of GDNF expression (Chen and Liu, 2016). In this study, we explored this question using *Fshb* and *Lhcgr* KO mice and dissected the role of gonadotropic pituitary hormones in SSC regulation.

Analysis of *Fshb* KO mice showed that SSCs in these animals are apparently normal. Immunohistochemical staining indicated that the numbers of $GFRA1^+$ spermatogonia and undifferentiated spermatogonia were not significantly different from those in WT mice. Because a positive relationship between FSH receptor and GDNF expression was reported (Ding et al., 2011; Tadokoro et al., 2002), we expected that SSCs would be significantly reduced in *Fshb* KO mice. However, *Fshb* KO mice contained a normal number of SSCs, suggesting that the lack of FSH signaling does not influence SSCs. These results were in contrast to previous studies that showed the beneficial effects of FSH on GDNF expression (Ding et al., 2011; Tadokoro et al., 2002). At least two explanations may explain this difference. The first is the method of FSH suppression. Although GnRH treatment was shown to increase GDNF expression in vivo via FSH suppression (Tadokoro et al., 2002), this treatment also decreases LH levels, which were not investigated in the previous report because of the lack of effect of testosterone on GDNF expression in vitro. The second is the experimental method; several studies showing increased GDNF expression were based on testis cell culture. However, FSH receptor expression levels may have changed by dissociation into single cells, and the continuous exposure to high levels of FSH in vitro may not have reflected physiological regulation of GDNF.

Transplantation of *Lhcgr* KO mouse testis resulted in enhanced colonization of adult donor cells. *Lhcgr* KO mice are characterized by immature Leydig cells and lack of Leydig stem cells (Lei et al., 2001; Lo et al., 2004). Leydig cells in *Lhcgr* KO mice are of the fetal type and the

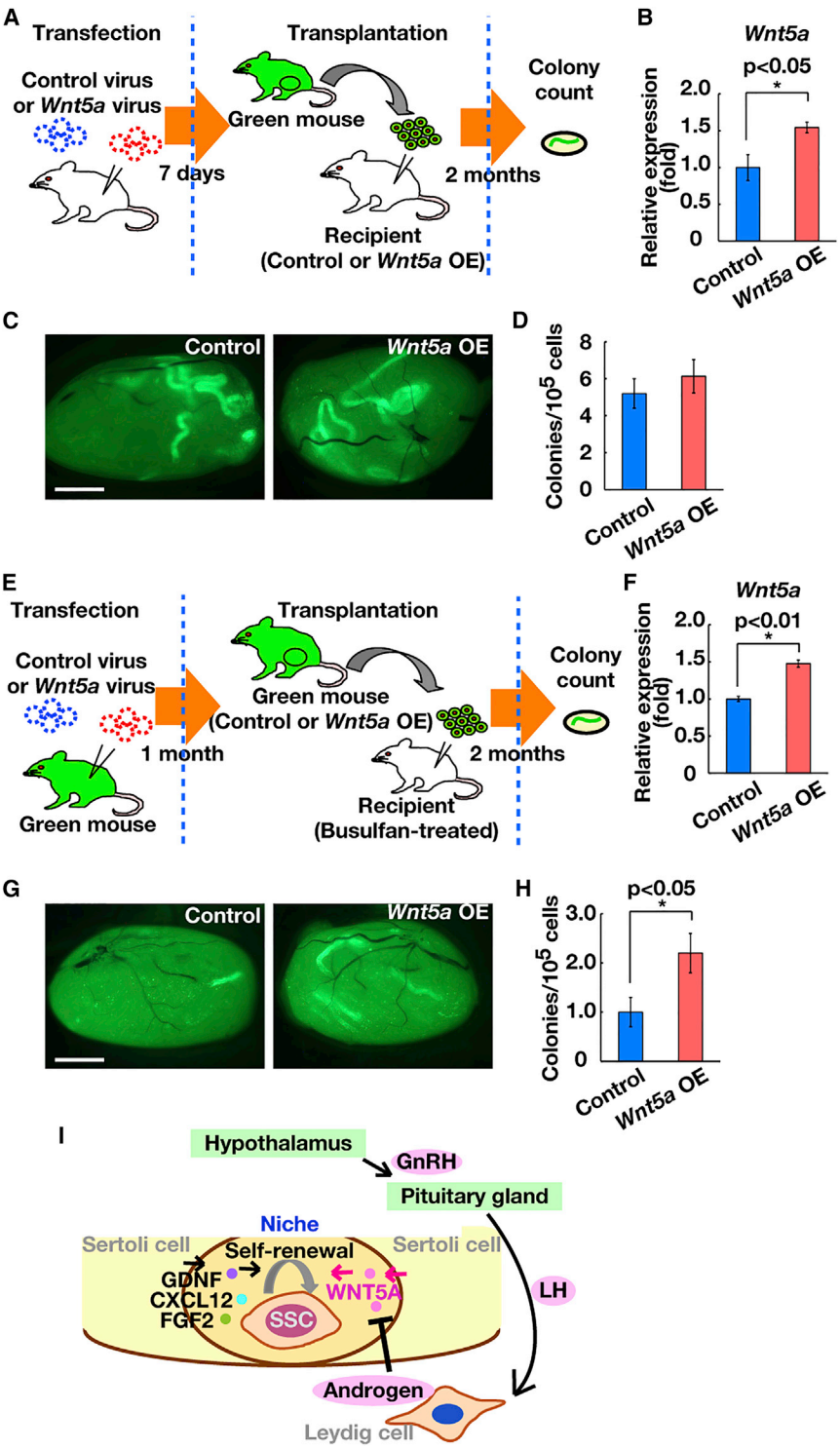

Figure 6. Increased SSC Activity by *Wnt5a* Treatment In Vivo

(A) Experimental strategy to quantify SSCs after *Wnt5a* OE in recipient testes.

(B) Real-time PCR analysis of *Wnt5a* expression in busulfan-treated testes 4 days after *Wnt5a* OE (n = 4 experiments).

(C) Macroscopic appearance of recipient testes injected with a *Wnt5a*-expressing lentivirus prior to donor cell transplantation.

(D) Colony counts (n = 8 testes for *Wnt5a* OE, n = 10 testes for control; four experiments).

(E) Experimental strategy to quantify SSCs after *Wnt5a* OE in donor green mouse testes.

(F) Real-time PCR analysis of *Wnt5a* expression in green mouse testes 4 days after *Wnt5a* OE (n = 3 experiments).

(G) Macroscopic appearance of recipient testes that received transplantation of green mouse testis injected with a *Wnt5a*-expressing lentivirus.

(H) Colony counts (n = 18 testes for *Wnt5a* OE, n = 19 testes for control; three experiments).

(I) Summary figure. Androgen secreted from Leydig cells via LH signal suppresses the WNT5A expression from Sertoli cells and modulates SSC self-renewal.

Scale bars represent 1 mm (C and G). Error bars indicate SEM. Asterisks indicate statistical significance. See also Table S2.

concentration of testosterone is very low (Zhang et al., 2001). Because it has been suggested that Leydig cells contribute to SSC niches and a recent study showed positive regulation of GDNF by testosterone (Chen et al., 2014; Oatley et al., 2009), we expected that SSCs in these mice might be decreased due to the lack of adult-type Leydig cells. However, we found an increased number not only of GFRA1-expressing spermatogonia but also SSCs. While these results suggest that SSC activity is not compromised by the lack of mature Leydig cells or testosterone, it remains

possible that the enrichment of SSCs was due to the absence of differentiating germ cells.

This hypothesis was tested by serial transplantation experiments, in which WT SSCs were transplanted into germ cell-deficient *Lhcgr* KO microenvironment. Although we did not observe an increase in colony number when *Lhcgr* KO mice were used as recipients, the number of secondary colonies increased in secondary recipients. Because an increased colony number in secondary recipients suggests enhanced self-renewal division in primary recipients, our results suggest that the absence of LH does not influence SSC homing but increases self-renewal divisions. Although this result demonstrated that LH suppresses proliferation of SSCs, it did not agree with our previous study that showed both increased colony numbers and self-renewal divisions in hypophysectomized adult recipients (Kanatsu-Shinohara et al., 2004). These results suggest that increased colonization and enhancement of SSC self-renewal are two separate phenomena, the latter of which is caused by the suppression of LH.

Our studies strongly suggest that *Wnt5a* upregulation is responsible for increased SSC activity in *Lhcgr* KO mice. The beneficial effects of *Wnt5a* were reported in a recent study (Yeh et al., 2011) in which *Wnt5a* increased the survival of SSCs in vitro. Although this study suggested that suppression of apoptosis is the primary role of *Wnt5a*, a net increase in the SSC number by *Wnt5a* was not demonstrated. Moreover, *Wnt5a* expression was examined only by measuring *Wnt5a* mRNA levels via in situ hybridization, and the role of *Wnt5a* in vivo remains unclear. In contrast, we showed that *Wnt5a* increases the number of SSCs both in vitro and in vivo. Because the effect of *Wnt5a* OE on SSC increase was modest compared with *Lhcgr* KO mice, it remains possible that other factors are involved. While we cannot exclude the possibility that WNT5A may not have been overexpressed in all cells, these results strongly suggest that *Wnt5a* is responsible, at least in part, for enhanced SSC self-renewal in *Lhcgr* KO mice. Because WNT5A is expressed more strongly in pup testes, it may be one of the factors that contribute to the rapid increase in SSC numbers during perinatal testis development (Shinohara et al., 2001). In fact, the plasma concentration of LH in prepubertal mice is relatively low until 7–8 days of age (Michael et al., 1980), which may induce WNT5A expression. The lack of LH appears to partially induce an immature phenotype in Sertoli cells because AMH immunoreactivity also increased in mature *Lhcgr* KO mice. Therefore, LH is not only required for Leydig cell maturation but is also important for suppressing the immature phenotype in Sertoli cells, although this effect of LH on Sertoli cells is probably indirect and most likely involves testosterone.

At least two important questions arise from the current study. First, how GDNF is regulated remains unclear. Although it was recently proposed that androgens regulate GDNF expression in peritubular cells (Chen et al., 2014, 2016), the effect of testosterone was not examined in vivo in these studies and we did not observe significant changes in GDNF expression in *Lhcgr* KO mice. We also failed to induce changes in GDNF levels by flutamide treatment, which suggests that GDNF is not regulated by androgens. However, caution is necessary in the latter experiment because it remains possible that flutamide treatment may induce testosterone production via an LH increase in normal mice. Identification of positive and negative regulators of GDNF expression and understanding how they are coordinated GDNF expression are urgent problems in understanding niche function. Second, it is important to identify the signal that enhances SSC colonization in GnRH-treated recipients or hypophysectomized animals. Although increases in colony numbers were observed in GnRH-treated and hypophysectomized mice, respectively (Dobrinski et al., 2001; Kanatsu-Shinohara et al., 2004; Ogawa et al., 1998), we did not observe an apparent increase in the number of colonies in *Lhcgr* KO recipients. Therefore, pituitary signals other than LH are likely responsible for the enhanced colonization of donor cells. In this context it should be noted here that, although GnRH antagonist treatment before the transplantation improved colonization, the same treatment did not enhance colonization when GnRH was administered at the time of transplantation (Dobrinski et al., 2001). It is believed that the major effect of GnRH antagonist is primarily on the homing step (Wang et al., 2010). Such differences between our current results and previous studies may be due to *Lhcgr* KO mice having cryptorchid testes, which might have influenced homing efficiency. Understanding the mechanism underlying the difference between GnRH treatment and *Lhcgr* KO mice may lead to the development of improved techniques to increase spermatogonial transplantation efficiency.

Although it is believed that gonadotropic pituitary hormones regulate GDNF expression in the testes, our study showed that neither FSH nor LH regulates GDNF expression. Instead, we found that increased WNT5A by LH suppression is responsible for enhanced SSC self-renewal. This suppression of WNT5A was likely mediated by androgens because flutamide also increased WNT5A expression. We also suggest that enhancement of colonization and increased self-renewal divisions are two distinct phenomena regulated by gonadotropic pituitary hormones. Considering that Sertoli and Leydig cell division is influenced by gonadotropic pituitary hormones, it is not surprising that complex regulations and interactions exist between the testis and pituitary gland. Future studies should explore these interactions, which not only increase our understanding of interorgan interactions to regulate

SSCs, but may provide efficient methods for improving spermatogonial transplantation, manipulating the amount of sperm production, and developing male contraceptives.

EXPERIMENTAL PROCEDURES

Animals

Fshb KO mice were purchased from the Jackson Laboratory (B6; 129S7-*Fshb*^{tm1Zuk}/J). *Lhcgr* KO mice were generated previously (Lei et al., 2001). The genotypes of the mice were examined by PCR with the primers listed in Table S2. We also used the transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01 (designated green; a gift from Dr. M. Okabe, Osaka University) to introduce the *Egfp* transgene as a donor cell marker. Dissociated testis cells were transplanted into W mice (Japan SLC) to quantify SSCs in KO mouse testes. For the remaining transplantation experiments, we used C57BL/6 × DBA/2 F1 (BDF1) mice that had been treated with busulfan (44 mg/kg). For the serial transplantation experiments, testis cells were transplanted into KO or control mice that had been treated with busulfan (44 mg/kg). All busulfan-treated recipient mice were used 4 weeks after busulfan treatment. Where indicated, animals were treated with flutamide (125 mg per mouse subcutaneously; Pfizer). The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols.

Statistical Analyses

Significant differences between means for single comparisons were determined by two-tailed Student's t test. For comparison of ratios during serial transplantation, we used one-tailed Student's t test. Multiple comparison analyses were performed using ANOVA followed by Tukey's honestly significant difference.

ACCESSION NUMBERS

Raw datasets have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) and are available under the accession number GEO: GSE77500 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77500).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.07.005.

AUTHOR CONTRIBUTIONS

T.T. and T.S. designed research, carried out experiments, and analyzed data. Z.L. and C.V.R. provided research material. T.T., M.K.-S., and T.S. wrote the paper.

ACKNOWLEDGMENTS

We thank Ms. Y. Ogata for technical assistance. Financial support for this research was provided by the Uehara Memorial Foundation, Takeda Science Foundation, the Naito Foundation, the Ministry of Education, Culture, Sports, Science, and Technology of Japan (15K08153), and the Japan Science and Technology Agency (PRESTO), Grants-in-aid for Scientific Research on Innovative Areas "Epigenome Dynamics and Regulation in Germ Cells" (25112003) and "Stem Cell Aging and Disease" (15H01510).

Received: May 6, 2016
Revised: July 7, 2016
Accepted: July 8, 2016
Published: August 9, 2016

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